Protocol for Measurement of Functional Anti-Drug Antibodies (ADA) Against Broadly Neutralizing Antibodies (bnAbs) in the TZM-bl Assay

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I. Introduction

Several broadly neutralizing antibodies (bnAbs) are in clinical development for the prevention and treatment of HIV-1 infection. Though originating in humans, their passive delivery has potential to induce anti-drug antibodies (ADA) that can interfere with the neutralizing activity of the bnAb. This protocol describes a modification of the TZM-bl assay that permits the quantitative detection of ADA. The assay measures a reduction of bnAb neutralizing activity as a function of Tat-induced luciferase (Luc) reporter gene expression after a single round of virus infection. TZM-bl cells (also called JC57BL-13) may be obtained from the National Institutes of Health (NIH) AIDS Reagent Program. This is a HeLa cell clone that was engineered to express CD4 and CCR5 [7.1] and contains integrated reporter genes for firefly luciferase and *E. coli* β-galactosidase under control of an HIV-1 LTR [7.2], permitting sensitive and accurate measurements of infection. The cells are highly permissive to infection by most strains of HIV, SIV and SHIV, including primary HIV-1 isolates and Env-pseudotyped viruses. DEAE dextran is used in the medium during neutralization assays to enhance infectivity. Expression of the reporter genes is induced in trans by viral Tat protein soon after infection. Luciferase activity is quantified by luminescence and is directly proportional to the number of infectious virus particles present in the initial inoculum. The assay is performed in 96-well culture plates for high throughput capacity. Use of a clonal cell population provides enhanced precision and uniformity. The TZM-bl assay has been validated for single-round infection with either uncloned viruses grown in human lymphocytes or Env-pseudotyped viruses produced by transfection in 293T/17 cells [7.3]. For measurements of ADA, serum, plasma, or specially processed samples with remaining bnAb(s) removed are assayed in the presence of a single fixed concentration of bnAb. Results are expressed as a reduction in bnAb neutralizing activity. This functional anti-drug antibody (ADA) assay for HIV-1 bnAbs has been optimized and qualified [7.4].

II. Definitions

%CV: Percent coefficient of variation

ADA: anti-drug antibodies

bnAbs: broadly neutralizing antibodies

DEAE-Dextran: Diethylaminoethyl-Dextran

DPBS: Dulbecco’s Phosphate Buffered Saline

EDTA: Ethylenediaminetetraacetic acid

GM: Complete Growth Medium

ID: Identification

Luc: Luciferase

NIH: National Institutes of Health

PI: Principal Investigator

PPE: Personal Protective Equipment

RLU: Relative Luminescence Units

TCID: Tissue Culture Infectious Dose

Vol: Volume

III. Reagents and Materials

Recommended vendors are listed. Unless otherwise specified, products of equal or better quality than the recommended ones can be used whenever necessary.

TZM-bl Cells
Vendor: NIH AIDS Reagent Program

Complete Growth Medium (GM) (see Protocol for Reagent Preparation for Use in the Neutralizing Antibody Assay for HIV-1 in TZM-bl Cells)

DEAE-Dextran, hydrochloride, average Mol. Wt. 500,000 (see Protocol for Reagent Preparation for Use in the Neutralizing Antibody Assay for HIV-1 in TZM-bl Cells)
Manufacturer: Sigma

Trypsin-EDTA (0.25% trypsin, 1 mM EDTA, sterile) (see Protocol for Thawing, Expanding, Maintaining, and Cryopreserving Adherent Cell Lines)
Manufacturer: Thermo Fisher Scientific

Trypan Blue (0.4%)
Manufacturer: Thermo Fisher Scientific

Dulbecco's Phosphate Buffered Saline (DPBS), sterile
Manufacturer: Thermo Fisher Scientific

Britelite™ plus Reporter Gene Assay System (see Protocol for Reagent Preparation for Use in the Neutralizing Antibody Assay for HIV-1 in TZM-bl Cells**)**
Manufacturer: PerkinElmer, Inc.

NOTE 1: The lyophilized britelite™ plus substrate is not classified as hazardous.

Bright-Glo™
Manufacturer: Promega

NOTE 2: Bright-Glo™ Luciferase Assay System from Promega Corporation is an acceptable substitute for britelite™ plus. Please follow manufacturer's guidelines for preparation and use. Bright-Glo™ is classified as hazardous. Personal Protective Equipment (PPE) is required when working with this reagent.

Microliter pipettor tips, sterile
Manufacturer: Rainin or Sartorius

Disposable pipettes, sterile, individually wrapped
1 ml pipettes
2 ml pipettes
5 ml pipettes
10 ml pipettes
25 ml pipettes
50 ml pipettes
100 ml pipettes
Manufacturer: Corning

Flat-bottom culture plates, 96-well, low evaporation, sterile
Manufacturer: Corning

Flat-bottom black solid plates, 96-well
Manufacturer: PerkinElmer, Inc.

Culture flasks with vented caps, sterile
Manufacturer: Corning
T-25 flask
T-75 flask

Reagent reservoirs, 50 ml, 100 ml capacity
Manufacturer: Corning

IV. Instrumentation

Recommended manufacturers are listed. Unless otherwise specified, equipment of equal or better quality than the recommended ones can be used whenever necessary.

Biological Safety Cabinet
Manufacturer: Baker Co.

Incubator (37°C, 5% CO2 standard requirements)
Manufacturer: Panasonic

Centrifuge and Microcentrifuge
(low speed centrifuge capable of up to 500 x g)
50 ml tube holder
15 ml tube holder
Manufacturer: Eppendorf

Microtitration plate holder
Manufacturer: Jouan

18 place standard rotor F-45-18-11 for 1.5 ml microcentrifuge tubes
Manufacturer: Eppendorf

Luminometer
Manufacturer: PerkinElmer, Inc.

Computer

Manufacturer: Dell

Water bath
Manufacturer: VWR International

Hemacytometer
Manufacturer: INCYTO

NOTE 3: An automated cell counting device (e.g., Countess, Manufacturer: Invitrogen) may be used in lieu of a light microscope / hemacytometer for cell counting and viability calculation.

Pipettor
Single channel electronic pipettor, 10-300 µl
12-channel electronic pipettor, 50-1200 µl
12-channel electronic pipettor, 10-300 µl
Single channel manual, 0.5-10 µl
Single channel manual, 2-20 µl
Single channel manual, 20-200 µl
Single channel manual, 100-1000 µl
Manufacturer: Sartorius

PipetteAid XP
Manufacturer: Drummond Scientific Co.

12 channel pipettor, 20-200 µl
Manufacturer: Rainin

Light Microscope
Manufacturer: Olympus

Ultra Low Temperature Freezer (−70°C or lower)
Manufacturer: Harris or Thermo Fisher Scientific

4°C Refrigerator
Manufacturer: LABREPCO, Inc.

−20°C Freezer
Manufacturer: LABREPCO, Inc.

V. Specimens

Samples should be heat-inactivated at 56˚C as described in Protocol for Heat Inactivation of Serum and Plasma Samples. Samples may be serum or plasma, although serum is preferred. Anticoagulants in plasma are problematic in the assay, especially when heparin is used because some forms of heparin have potent and strain-specific antiviral activity. All anticoagulants are toxic to the cells at low plasma dilutions. Monoclonal antibodies should not be heat-inactivated.

VI. Protocol

1. Determine bnAb Concentration to be Used in the ADA Assay

1.1 Follow Protocol for the Neutralizing Antibody Assay for HIV-1 in TZM-bl Cells to determine the concentration of the bnAb that will be used in the ADA assay to yield 40-80% neutralization against the test virus.

2. ADA Assay in TZM-bl Cells Setup

NOTE 4: All incubations are performed in a humidified 37°C, 5% CO_2 incubator unless otherwise specified.

2.1. Prepare an adequate volume of GM+bnAb for the number of assays to be conducted. This GM+bnAb should contain a concentration of bNAb that results in 40-80% neutralization for the virus to be assayed. If the stock concentration of the bNAb is high, make a working stock with lower concentration so that the amount of working stock added to GM+bNAb for testing one plate is at least 5µl. Dilute the working stock of antibody in GM.

2.2. Using the format of a 96-well flat bottom culture plate as illustrated in Figure # 1, place 150 µl of regular GM (no bnAb present) in all wells of column 1 (cell control). Place 100µl of regular GM (no bnAb present) in all wells of column 2 (virus control). Place 200 – 250 µl of regular GM (no bnAb present) in all wells of column 12 (media blank). Place 100 µl of GM+bnAb in all wells of columns 3-11. Place an additional 40 µl of GM+bnAb in the bottom wells (row H) of columns 3-10.

Figure #1: Assay template for measuring ADA, 4 samples per plate

	1	2	3	4	5	6	7	8	9	10	11	12
A	CC	VC	Dil 8	Dil 8	Dil 8	Dil 8	Dil 8	Dil 8	Dil 8	Dil 8	BNC	MB
B	CC	VC	Dil 7	Dil 7	Dil 7	Dil 7	Dil 7	Dil 7	Dil 7	Dil 7	BNC	MB
C	CC	VC	Dil 6	Dil 6	Dil 6	Dil 6	Dil 6	Dil 6	Dil 6	Dil 6	BNC	MB
D	CC	VC	Dil 5	Dil 5	Dil 5	Dil 5	Dil 5	Dil 5	Dil 5	Dil 5	BNC	MB
E	CC	VC	Dil 4	Dil 4	Dil 4	Dil 4	Dil 4	Dil 4	Dil 4	Dil 4	BNC	MB
F	CC	VC	Dil 3	Dil 3	Dil 3	Dil 3	Dil 3	Dil 3	Dil 3	Dil 3	BNC	MB
G	CC	VC	Dil 2	Dil 2	Dil 2	Dil 2	Dil 2	Dil 2	Dil 2	Dil 2	BNC	MB
H	CC	VC	Dil 1	Dil 1	Dil 1	Dil 1	Dil 1	Dil 1	Dil 1	Dil 1	BNC	MB
			Sample 1		*Sample 2*		*Sample 3*		*Sample 4*			

CC = cell control wells (cells only)
VC = virus control wells (virus + cells only)
BNC = bnAb control wells (cells + virus + bN=nAb)
MB = media blank (media only)

NOTE 5: The difference in RLU between columns 2 and 11 provides a measure of the percent neutralizing activity of bnAb in the absence of ADA. The average RLU in column 11 (bnAb control) should reflect a 40-80% reduction (i.e., neutralization) of the average RLU in column 2 (virus control) for the assay to be acceptable. The average RLU in column 2 (virus control) should be ≥10X the average RLU in column 1 (cell control).

2.3. Centrifuge the test samples at the appropriate time and speed in order to pellet any debris that might be present.

2.4. Add 11 µl of test samples in duplicate to row H, columns 3-10 in following order: sample 1 – wells H3-H4, sample 2 – wells H5-H6, sample 3 – wells H7-H8, and sample 4 – wells H9-H10. Mix the samples (at least 4 times) in row H and transfer 50 µl to row G. Repeat the mixing and transferring of samples through row A (these are serial 3-fold dilutions). After final transfer and mixing is complete, discard 50 µl from the wells in columns 3-10, row A into waste container.

NOTE 6: This format is designed to assay 4 samples in duplicate wells at each serum dilution per plate (Figure #1). The above description is for a starting dilution of 1:20. This format is designed to measure ADA titers in the range of 1:20 to 1:43,740.

NOTE 7: Appropriate adjustments may be made to test a different range of dilutions (Figure #2). If using a different range of sample dilutions, for example 1:30 to 1:2,343,750 (1:30; 5 fold), it is advised to mix samples more than 4 times before each transfer.

Figure #2: Sample dilution charts

STANDARD DILUTION CHART FOR 2-FOLD SAMPLE DILUTIONS

Desired Start Dilution	GM + bnAb Volume (µl)	Sample Volume (µL)
1:5	40	60
1:10	70	30
1:15	80	20
1:20	85	15
1:25	90	12
1:30	90	10
1:50	95	6

Place 150 µl of regular GM (no bnAb present) in all wells of column 1 (cell control). Place 100 µl of regular GM (no bnAb) in all well of column 2 (virus control). Place 200-250 µl of regular GM (no bAb) in all wells of column 12 (media blank). Then place 100 µl of GM+bnAb in all wells of columns 3-11. Add extra amount of GM+bnAb listed above in the bottom wells (row H) of columns 3-10. Then add the corresponding sample volume to the bottom wells (row H) and do 2-fold dilutions (i.e., serial transfers of 100 µl).

STANDARD DILUTION CHART FOR 3-FOLD SAMPLE DILUTIONS

Desired Start Dilution	GM + bnAb Volume (µl)	Sample Volume (µL)
1:5	5	45
1:8	25	28
1:10	30	22
1:15	35	15
1:20	40	11
1:24	50	10
1:30	42.5	7.5
1:45	45	5

Place 150 µl of regular GM (no bnAb present) in all wells of column 1 (cell control). Place 100 µl of regular GM (no bnAb) in all well of column 2 (virus control). Place 200-250 µl of regular GM (no bnAb) in all wells of column 12 (media blank). Then place 100 µl of GM+bnAb in all wells of columns 3-11. Add extra amount of GM+bnAb listed above in the bottom wells (row H) of columns 3-10. Then add the corresponding sample volume to the bottom wells (row H) and do 3-fold dilutions (i.e., serial transfers of 50 µl).

STANDARD DILUTION CHART FOR 5-FOLD SAMPLE DILUTIONS

Desired Start Dilution	GM + bnAb Volume (µl)	Sample Volume (µL)
1:10	7	18.75
1:15	12.5	12.5
1:20	15	9.5
1:25	17.5	7.5
1:30	20	6.25
1:50	21	3.75

Place 150 µl of regular GM (no bnAb present) in all wells of column 1 (cell control). Place 100 µl of regular GM (no bnAb) in all well of column 2 (virus control). Place 200-250 µl of regular GM (no bnAb) in all wells of column 12 (media blank). Then place 100 µl of GM+bnAb in all wells of columns 3-11. Add extra amount of GM+bnAb listed above in the bottom wells (row H) of columns 3-10. Then add the corresponding sample volume to the bottom wells (row H) and do 5-fold dilutions (i.e., serial transfers of 25 µl).

NOTE 8: A positive control with a known ADA titer against the bnAb/target virus combination should be included on at least one plate in series each time assays are performed.

2.5. Thaw the required number of vials of virus by placing in an ambient temperature water bath. When completely thawed, mix the virus in the tube (do not vortex), and then dilute the virus in GM (no bnAb present) to achieve a TCID of approximately 50,000-150,000 Relative Luminescence Units (RLU) equivalents (+/- 15,000 RLU). For pseudoviruses that do not reach 50,000-150,000 RLU, pick a dose of virus that gives at least 10 times the background RLU but is not toxic to the cells (observed via light microscopy). See Protocol for the Preparation and Titration of HIV-1 Env-Pseudotyped Viruses.

NOTE 9: The RLU equivalents measured in the TCID assay may not match the RLUs in the virus control (column 2) of the neutralization plate. This difference is acceptable provided that the average RLU value in column 2 (virus control) is ≥10X the average RLU value in column 1 (cell control) and the virus control is not toxic to the cells observed by light microscopy.

NOTE 10: Leftover virus may be refrozen in an ultra low temperature freezer and marked with a "1X" on the lid and label of the vial. The "1X" notes that that particular vial has been thawed one time. When using "1X" vials of virus in the TZM-bl assay, the technician must consult the virus database to obtain the optimal virus dilution for viruses that have been thawed one time. No pseudovirus should be used in the TZM-bl assay if it has been thawed and refrozen more than once.

NOTE 11: For viruses with high dilution (i.e., 1:100 or higher), it is recommended to aliquot and freeze leftover virus into smaller volumes. The aliquots should be based on the recommended dilution determined from the TCID assay of a 1X thawed virus aliquot (see Protocol for the Preparation and Titration of HIV-1 Env-Pseudotyped Viruses). If no dilution is given for a 1X thawed virus, make 150-300 µl aliquots. The following information should be written on the 1X tube: name of the virus, harvest date, and volume of virus. A "1X" should be written on the cap of the tube and the tube to denote that the virus has been thawed and refrozen once.

2.6. Prepare virus/GM suspension at the recommended dilution as described below:

2.6.1. Virus Calculations:

To calculate the total volume (vol.) of virus/GM mixture needed for the assay, multiply the total number of plates by the volume of virus/GM mixture to be used per plate. Then

divide the total volume of virus/GM mixture by the optimal virus dilution to use (based on the TCID assay) to derive the volume of undiluted virus needed. Then subtract the volume of undiluted virus needed from the total volume of virus/GM mixture to derive the volume of GM needed.

Total number of plates X Vol. of virus/GM per plate = Total vol. virus/GM needed

Total vol. virus/GM needed ÷ Optimal virus dilution = Vol. of undiluted virus needed

Total vol. of virus/GM needed – Vol. of undiluted virus needed = Vol. of GM needed

2.6.2. The virus/GM suspension should be prepared as follows: Add GM and virus and thoroughly mix immediately prior to plating. Dispense 50 µl of the virus/GM suspension to all wells in columns 2-11, from rows A to H. Columns 1 and 12 will not receive virus.

2.7. Cover the plates and incubate for 45 – 90 minutes.

2.8. During the incubation, prepare a suspension of TZM-bl cells at a concentration of 100,000 cells/ml in GM (no bnAb present) as described below:

2.8.1. Perform Viable Cell Count (see site-specific protocol)

2.8.2. Cell Calculations (if using a hemacytometer):
To calculate the cell concentration, multiply the average number of cells per quadrant, the dilution factor, and 10,000 to yield the cell concentration, "C_1", in cells/ml. To calculate the total cell mixture volume, "V_2", that you need, multiply the number of plates by the total volume of cell mixture needed per plate. The concentration of cells desired is 100,000 cells/ml, "C_2". Thus, using the equation $C_1V_1 = C_2V_2$, one can solve for "V_1", the volume of cells needed.

For example:

Total number of viable cells counted = 60
Number of quadrants counted = 4
Dilution factor = 10
Number of plates = 1
Cell mixture needed per plate = 10 ml

60 cells ÷ 4 quadrants = 15 cells/quadrant

15 cells/quadrant x dilution factor of 10 x 10,000 cells/ml = 1,500,000 cells/ml;
$15 \times 10 \times 10^4 = 1.5 \times 10^6$ cells/ml = C_1

1 plate x 10 ml/plate = 10 ml = V_2

Optimum final concentration of cells = $1 \times 10^5 = C_2$

$C_1V_1 = C_2V_2$ Therefore: $V_1 = C_2V_2 \div C_1$
$(1 \times 10^5$ cells/ml x 10 ml) ÷ 1.5×10^6 cells/ml = 0.67 ml of cells

2.8.3. Addition of DEAE-Dextran to Cells

NOTE 12: The concentrations of DEAE-Dextran shown will vary by batch of DEAE-Dextran. The actual optimal concentration should be determined for each new batch of DEAE-Dextran prepared in accordance with Protocol for the Determination of Optimal Concentration of DEAE-Dextran.

To calculate the amount of DEAE-Dextran to use, first multiply the optimal concentration of DEAE-Dextran (see Protocol for Reagent Preparation for Use in the Neutralizing Antibody Assay for HIV-1 in TZM-bl Cells) by 0.250 ml (the final volume in each well) to get the amount of DEAE-Dextran per well. Multiply the amount of DEAE-Dextran per well by 100 wells/plate (96 wells rounds to 100) to derive the amount of DEAE-Dextran per plate. Divide the amount of DEAE-Dextran needed per plate by the stock concentration of the DEAE-Dextran to yield the volume of DEAE-Dextran stock needed. Multiply this number by the number of plates to yield the total volume of DEAE-Dextran stock needed.

For example:

If the optimal concentration of DEAE-Dextran in the assay is 10 µg/ml and the DEAE-Dextran stock is at 5 mg/ml:

10 µg/ml x 0.25 ml (volume in well) = 2.5 µg of DEAE-Dextran needed in each well

2.5 µg x 100 wells/plate = 250 µg of DEAE-Dextran needed per plate = 0.25 mg of DEAE-Dextran

0.25 mg of DEAE-Dextran per plate ÷ 5 mg/ml stock concentration = 0.05 ml of DEAE-Dextran stock needed per plate

To calculate the amount of Growth Medium to add, subtract the total volume of cells needed and the total volume of DEAE-Dextran stock needed from the total volume of cell mixture needed.

So, the total volume needed for one plate is 10 ml.

10 ml – 0.67 ml cells – 0.05 ml DEAE-Dextran = 9.3 ml of GM

2.9. The GM/cells/DEAE-Dextran suspension should be prepared as follows: Add GM and DEAE-Dextran then mix; Add cells and thoroughly mix the prepared cell suspension immediately prior to plating. Dispense 100 µl of the prepared cell suspension (10,000 cells per well) to each well in columns 1-11. Cells should be added to cell control wells first (on all plates) and then from row A to H.

NOTE 13: The use of DEAE-Dextran is optional. When omitted, the TCID of the virus should be measured in the absence of DEAE-Dextran.

2.10. Cover plates and incubate for 44-72 hours if Env-pseudotyped viruses are used. If replication-competent virus is used, the plates should be incubated for 44-50 hours to minimize virus replication.

2.11. After incubation, remove plates from the incubator. Plates should not stay out of the incubator longer than one hour before running the luciferase reaction.

NOTE 14: Using a microscope, examine at least 2 virus control wells (column 2) for the presence of syncytia. It is important to note the presence of syncytia as too many syncytia indicate cell killing and thus the validity of the assay is compromised. If cell killing is present, the assays should be repeated using a lower dose of the virus. Also check the plate for cell toxicity in the presence of samples at the lowest dilution (and higher dilutions if toxicity is observed). Cell toxicity could be erroneously interpreted as neutralization.

2.12. Thaw *britelite™ plus* or *Bright-Glo™* directly before use in an ambient temperature water bath away from light. Once thawed, invert tube a few times to mix.

2.13. Remove 150 µl of culture medium from each test well in columns 1-11, leaving approximately 100 µl.

2.14. Dispense 100 µl of *britelite™ plus* or *Bright-Glo™* to each test well in columns 1-11.

2.15. Incubate away from light at room temperature for 2 minutes (but no longer than 15 minutes) to allow complete cell lysis then mix by pipettor action (at least two strokes) and transfer 150 µl to a corresponding 96-well black plate. Read the plate in a luminometer.

NOTE 15: Bright-*Glo™* and *britelite™ plus* reagents can be subjected to 7 or 10 freeze thaw cycles respectively with no effect on potency.

3. Analyzing and printing results

3.1. Prior to reading the plates in the luminometer, enter the assay protocol information in the luminometer software.

3.2. Read the plates in a luminometer interfaced to a dedicated computer in the laboratory. Two data files are generated from the luminometer for each plate read: an Excel file and a non-modifiable file.

3.3. The software program associated with the luminometer automatically saves the raw data in Excel format in a desired location, after each plate is read, using a unique file identification number (ID) for each plate.

3.4. Save the original plate data file directly from the luminometer software as a PDF file or another non-modifiable file. This file should also be saved automatically in a read-only folder, for archival purpose.

3.5. Analyze and print the data using the Microsoft Excel "ADA macro" provided by the Montefiori Laboratory.

NOTE 16: The ADA macro calculates the percent neutralization provided by each sample dilution. Percent neutralization is determined by calculating the difference in average RLU between virus control (cells + virus, column 2) and test wells (cells + sample + virus), dividing this result by the difference in average RLU between virus control (cells + virus, column 2) and bnAb control wells (cells + bnAb + virus, column 11), and multiplying by 100. ADA titers are expressed as the reciprocal of the sample dilution required to decrease the neutralizing activity of the bnAb below 50%. Failure to score a value below 50% neutralization at any sample dilution constitutes a negative test.

3.6. Prepare a printed data packet to provide to the final reviewer and/or PI.

NOTE 17: Based upon the PI's request, a copy of the original plate data (saved as a non-modifiable file) may be kept in electronic form.

4. Pass/Fail Criteria

4.1. The average RLU of the virus control wells is ≥10X the average RLU of cell control wells.

4.2. The average RLU of the bnAb control wells is 40-80% of the average RLU of the virus control wells.

4.3. The percent coefficient of variation (%CV) between RLU in the virus control wells is ≤30%.

4.4. The percent coefficient of variation (%CV) between RLU in the bnAb control wells is ≤30%.

4.5. The percent difference between duplicate wells is ≤30% for sample dilutions that yield at least 40% neutralization.

4.6. Neutralization curves cross the 50% neutralization cut-off 0-1 times.

4.7. The value of the positive control is within a 3-fold range of previous values for that particular control-virus combination.

VII. References

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